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Thioredoxin 1 Promotes Intracellular Replication and Virulence of *Salmonella enterica* Serovar Typhimurium

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The effect of the cytoplasmic reductase and protein chaperone thioredoxin 1 on the virulence of *Salmonella enterica* serovar Typhimurium was evaluated by deleting the *trxA*, *trxB*, or *trxC* gene of the cellular thioredoxin system, the *grxA* or *gshA* gene of the glutathione/glutaredoxin system, or the *dsbC* gene coding for a thioredoxin-dependent periplasmic disulfide bond isomerase. Mutants were tested for tolerance to oxidative and nitric oxide donor substances in vitro, for invasion and intracellular replication in cultured epithelial and macrophage-like cells, and for virulence in BALB/c mice. In these experiments only the *gshA* mutant, which was defective in glutathione synthesis, exhibited sensitization to oxidative stress in vitro and a small decrease in virulence. In contrast, the *trxA* mutant did not exhibit any growth defects or decreased tolerance to oxidative or nitric oxide stress in vitro, yet there were pronounced decreases in intracellular replication and mouse virulence. Complementation analyses using defined catalytic variants of thioredoxin 1 showed that there is a direct correlation between the redox potential of thioredoxin 1 and restoration of intracellular replication of the *trxA* mutant. Attenuation of mouse virulence that was caused by a deficiency in thioredoxin 1 was restored by expression of wild-type thioredoxin 1 in *trans* but not by expression of a catalytically inactive variant. These results clearly imply that in *S. enterica* serovar Typhimurium, the redox-active protein thioredoxin 1 promotes virulence, whereas in vitro tolerance to oxidative stress depends on production of glutathione.

Production of chemical radicals upon microbial contact is a central and efficient innate antimicrobial defense mechanism of mammalian phagocytic cells. The importance of radical production is illustrated by the increased susceptibility to infection of individuals defective in functional phagocyte NADPH oxidase, which is responsible for the production of oxygen radicals (41); it is also shown by the multiple protective mechanisms used by microbes to scavenge or counteract radical stress (9, 24, 51, 52, 54). *Escherichia coli* uses two different defined pathways for protective responses against oxidative stress. One pathway relies on the OxyR transcriptional regulator, a member of the LysR/MetR family of prokaryotic gene regulatory proteins, which has the intrinsic ability to sense redox stress (2). OxyR activates genes coding for alkyl hydroperoxide reductase and catalase hydroperoxide I in response to either H_2O_2 or a decreased intracellular thiol/disulfide ratio (59). SoxRS, which constitutes the second system, is activated by superoxide and/or nitric oxide stress and induces expression of superoxide dismutase and glucose-6-phosphate dehydrogenase (21).

When selected oxidative substances, such as H_2O_2, are produced at an extracellular location, they may move through membrane structures and affect the redox status of cytosolic proteins and oxidize cysteine sulfhydryl side chains essential for protein folding, for enzymatic activity, or for chelating essential metal cofactors (3). The *E. coli* thioredoxin and glutathione/glutaredoxin systems include enzymes which shuttle redox potential from NADPH to cytosolic substrates, thereby providing a repair system for oxidized sulfhydryl groups in cytosolic proteins (3, 4, 50) (Fig. 1). Using thioredoxin 1 (TrxA) and thioredoxin 2 (TrxC), the thioredoxin system transfers reducing equivalents to DsbD, which is located in the cytoplasmic membrane, for transfer to the periplasmic space, thus providing the periplasmic oxidoreductase DsbC with electrons (27). DsbC, in turn, by reducing wrongly paired disulfides, plays an important role in ensuring correct protein folding of periplasmic proteins (42). Both thioredoxin 1 and thioredoxin 2 are in turn continuously reduced, mainly by thioredoxin reductase (TrxB), which derives its reducing equivalents from NADPH (3).

The glutathione/glutaredoxin system, which relies on glutathione as a redox shuttle from NADPH to oxidized targets, consists of the glutathione-synthesizing enzymes γ-glutamylcysteine synthetase (GshA) and glutathione synthetase (GshB), glutathione oxidoreductase (Gor), glutaredoxin 1 (GrxA), glutaredoxin 2 (GrxB), and glutaredoxin 3 (GrxC) (3). For *E. coli* it has been shown that mutations in single components of the thioredoxin or glutathione/glutaredoxin system do not have profound effects on viability during standard in vitro growth conditions (46, 47). However, when *trxA*, *trxC*, and *grxA* are deleted simultaneously, the combination is lethal (50). Hence, the thioredoxin and glutathione/glutaredoxin pathways could be envisioned to be parallel mechanisms for transfer of electrons.

Salmonella enterica serovar Typhimurium causes a systemic disease in mice that in many respects resembles human typhoid fever. In the murine infection, macrophages of the liver and spleen are believed to be a major niche for intracellular bacterial multiplication (11, 33). Furthermore, the inability of bac-

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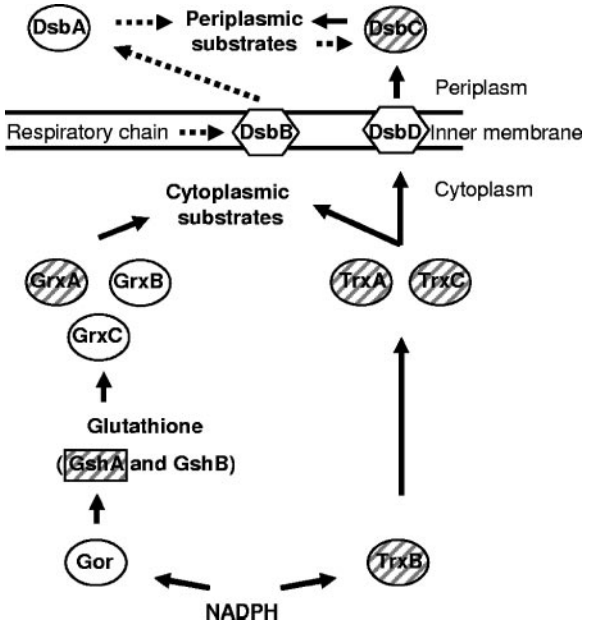

FIG. 1. Schematic overview of the thioredoxin and glutathione/glutaredoxin systems in *E. coli*. Reduction and oxidation are indicated by solid and dashed arrows, respectively. The double arrows indicate disulfide bond isomerization. Members of the systems studied in this paper are cross-hatched. The diagram was adapted from the work of Aslund and Beckwith (3).

teria to survive and replicate in macrophages or macrophage-like cell lines in vitro also correlates with decreased virulence (20). Several studies have indicated that phagocyte production of oxidative and nitrosative substances is a main effector in the innate defense against infection of murine primary macrophages and macrophage-like cells (17, 55), as well as mice (34, 49), by *S. enterica* serovar Typhimurium.

The annotated *S. enterica* serovar Typhimurium LT2 genome contains homologues of the genes encoding the *E. coli* glutathione/glutaredoxin and thioredoxin systems (3, 35), which implies that the redox-shuffling pathways that operate in *E. coli* may also function in salmonellae to provide protection against oxidative stress. Indeed, inactivation of *dsbA* and *srgA*, a plasmid-encoded paralogue of *dsbA*, in *S. enterica* serovar Typhimurium has been shown to affect the expression of virulence-associated type III secretion and plasmid-encoded fimbriae (8, 36). Yet the role of the thioredoxin and glutathione/glutaredoxin pathways, which provide the periplasmic DsbA and SrgA oxidoreductases with reducing capacity, in the fitness and virulence of *S. enterica* serovar Typhimurium has not been evaluated.

In this study we deleted individual genes coding for components of the thioredoxin pathway (*trxA*, encoding thioredoxin 1; *trxC*, encoding thioredoxin 2; *trxB*, encoding thioredoxin reductase), γ-glutamylcysteine synthetase (*gshA*), glutaredoxin 1 (*grxA*), and the periplasmic oxidoreductase DsbC (*dsbC*). Mutants were subsequently probed for their contributions of the genes to the in vitro and in vivo fitness and virulence of *S. enterica* serovar Typhimurium in epithelial MDCK cells, murine macrophage-like J774-A.1 cells, and BALB/c mice. The results define a demarcation in the biological functions of the two pathways in *S. enterica* serovar Typhimurium. Mutational depletion of bacterial glutathione, but not mutational depletion of glutathione reductase, resulted in sensitization to oxidative stress in vitro and in a slight attenuation of virulence. Depletion of thioredoxin 1, in turn, was specifically associated with decreased intracellular replication in both epithelial and macrophage-like cells and with drastically decreased virulence in BALB/c mice. Furthermore, our results clearly imply that the catalytic redox activity of thioredoxin 1 contributes to bacterial virulence.

MATERIALS AND METHODS

Bacterial strains, plasmids, and cultivation. This study was based on the virulent S. *enterica* serovar Typhimurium strain ATCC 14028 (ATCC, Manassas, VA). Bacteria were grown in either complex Luria-Bertani (LB) medium, minimal medium at pH 5.8 (MM5.8), or M9 minimal medium as described previously (26, 32, 58). When necessary, the growth media were supplemented with antibiotics (Sigma, St. Louis, MO) at the following concentrations; kanamycin, 30 μg/ml; tetracycline, 10 μg/ml; ampicillin, 100 μg/ml; and chloramphenicol, 10 μg/ml. In competition experiments LB broth was inoculated with a mixture of mutant and wild-type bacteria at a 1:1 ratio, and the inoculum contained 10^4 bacteria/ml. The cultures were plated on LB agar plates with and without kanamycin after 0 and 16 h of incubation at 37°C. A collection of *trxA* constructs, containing wild-type *trxA* or variants of *trxA* with different redox potentials under the control of the arabinose promoter in pBAD33 (4, 39, 40), were used to complement the *trxA* mutant during growth inside J774-A.1 macrophage-like cells. As the *trxA* constructs were derived from *E. coli*, it should be mentioned that the primary structures of the *E. coli* and *S. enterica* serovar Typhimurium LT2 thioredoxin 1 proteins are identical (7, 35). To ensure that thioredoxin 1 was expressed, cell culture media were supplemented with L-arabinose (20 mM; Sigma) and chloramphenicol throughout the experiment. Plasmid pPIR-A was used in segregation experiments (44).

Generation of mutants. The open reading frames of the *trxA*, *trxB*, *trxC*, *gshA*, *grxA*, and *dsbC* genes were replaced by a kanamycin resistance cassette using the recombinase-assisted allelic replacement technology described by Datsenko and Wanner (16). Briefly, the kanamycin resistance cassette was PCR amplified from plasmid pKD3 using primers with 40- to 50-mer nucleotide extensions homologous to the 5′ and 3′ ends of the open reading frames to be deleted. The primers were obtained from Invitrogen and are described in Table 1. The PCR products were transferred to wild-type bacteria by electroporation according to the instructions provided by the manufacturer of the electroporation cuvettes (Bio-Rad). Allelic replacement was assisted and enhanced by the phage λ Red recombinase expressed in *trans*, and undesired recombination activity was prevented by expression of the λ Red recombinase from an arabinose-inducible promoter (16). Additionally, to exclude any possible secondary mutations created by recombinase activity, all mutations were transferred to a clean background using P22 *int* transduction (48). All mutants were verified by PCR amplification with one primer defining the replacement cassette (16) and another primer defining the adjacent retained genomic sequence, as shown in Table 2. All primers were designed using the *S. enterica* serovar Typhimurium LT2 whole-genome sequence annotation from Washington University, St. Louis, MO, and were purchased from Gibco. The kanamycin resistance cassette contains recognition sequences for the FLP recombinase and can therefore be easily eliminated by using a helper plasmid expressing the FLP recombinase. Excision of the resistance gene left only a short (85-bp) oligonucleotide "scar" and thus generated a nonpolar mutation (16). A nonpolar *trxA* mutant, designated Δ*trxA* to distinguish it from the *trxA*::Km mutant, was generated using this method, and in addition, P22 *int* transduction (48) of Δ*trxA* was used to generate a Δ*trxA trxC*::Km double mutant.

In vitro assays for assessing tolerance to oxidative and nitric oxide donor substances. The following chemicals were obtained from Sigma (St. Louis, MO): H_2O_2, paraquat, diethyl maleate (DEM), and *S*-nitrosoglutathione (GSNO). In these experiments, wild-type *S. enterica* serovar Typhimurium ATCC 14028 or defined mutants of this strain were propagated in either LB medium or MM5.8 until the logarithmic or stationary phase of growth was reached, as described by Ygberg et al. (58). Bacteria (10^4) were seeded in wells of round-bottom microtiter plates (Nunc, Denmark) containing one of the substances diluted in LB medium or MM5.8 with twofold dilution between steps. The plates were incubated at 37°C for 16 h, and the loss of a pellet at the bottom of the plate was used as the readout for the MIC.

TABLE 1. Primers used for mutagenesis

Gene	Primer	Homology[a]
trxA	5′-ATGCTACACCAACACGCCAGGCTTATTCCTGTGGAGTTATATG TGTAGGCTGGAGCTGCT	42 bp directly upstream of the ORF
	5′-TTAGCCAGCCACTTCGCCGTTTTTAAACAGCAGCAGAGTCGGC ATATGAATATCCTCCTTA	42 bp inside the ORF starting 60 bp from the 3′ end
trxB	5′-ATGCTATTACACATATTGTTAACAAAATCGTTATGCTCTTGTGT AGGCTGGAGCTGCTTC	40 bp located 63 to 103 bp transcriptionally upstream of the ORF
	5′-CTATTTGCTCGCGTCGGCCAGACCATCGAGATAGCGTTCGGCA TATGGATATCCTTA	Last 44 bp in the ORF
trxC	5′-ATGAACACCGTTTGTACCCATTGTCAGGCCATTAACCGCAGTG TAGGCTGGAGCTGCTTC	First 40 bp in the ORF
	5′-TTACAGCGCTTCGTTCAGCCAGCTGTCGAAAGGCGCTTTACAT ATGAATATCCTCCTTA	Last 40 bp in the ORF
grxA	5′-ATGTTTACTGTTATTTTTGGCCGTCCAGGGTGCCCTTATTGTGT AGGCTGGAGCTGCTTC	First 41 bp in the ORF
	5′-TCAGGCGAAAAGATTCAGATTTTCTTTTGCCCATGCTTCAAAA TCCGTACCATATGAATATCCTCCTTA	Last 50 bp in the ORF
gshA	5′-ATGCTCACCTTTATGCGCGATCTCCATCGGTATACGGCCAGGT GTAGGCTGGAGCTGCTTC	42 bp inside the ORF starting 276 bp from the 5′ end of the ORF
	5′-CGCATATGGCAGGTAGTACATGCCGCAGTCCGTTTTTTCAACA TATGAATATCCTCCTTA	41 bp inside the ORF starting 865 bp from the 3′ end of the ORF
dsbC	5′-ATGAAAAAGCGTTTTATGATGTTCACTTTACTGGCGGCGGGTG TAGGCTGGAGCTGCTTC	First 40 bp in the ORF
	5′-TTATTTACCACTGGTCTGTTTTTGATGCTCATCAAGAAACCATA TGAATATCCTCCTTA	Last 40 bp in the ORF

[a] ORF, open reading frame.

In vitro cell culture infection model. J774-A.1 cells (TIB 67; American Type Culture Collection, Rockville, MD) and MDCK cells (American Type Culture Collection, Rockville, MD) were cultivated in RPMI and Dulbecco modified Eagle medium (Gibco, Paisley, United Kingdom), respectively. The medium was supplemented with 10% fetal bovine serum (Gibco, Paisley, United Kingdom), L-glutamine (final concentration, 10 mM), HEPES (final concentration, 10 mM), and gentamicin (final concentration, 10 μg/ml). L-Glutamine, HEPES, and gentamicin were all obtained from Sigma, St. Louis, MO.

The bacteria used for infection of J774-A.1 cells were grown on LB agar plates overnight at 37°C to ensure that the phenotype was noninvasive (6). Prior to infection bacteria were suspended in phosphate-buffered saline and opsonized in vitro for 30 min at 37°C using 10% fresh preimmune serum from BALB/c mice. Next, bacteria were diluted in cell culture medium supplemented only with HEPES (final concentration, 10 mM) and subsequently were seeded on semiconfluent cells grown in 24-well plates.

To obtain phenotypically invasive bacteria for infection of MDCK cells, cultures were grown overnight (16 to 18 h at 37°C) in LB broth, diluted 1:10 in 4 ml of fresh medium, and grown in closed 15-ml Falcon tubes at 37°C on a Brunswick roller incubator for 2 h (6). Next, the bacteria were diluted directly in HEPES-supplemented cell culture medium. Cells were infected at a multiplicity of infection of 20:1 and centrifuged for 5 min at 1,500 rpm to facilitate infection. One hour postinfection, medium containing gentamicin at a concentration of 50 μg/ml was added, and the cells were incubated for 45 min to kill the extracellular bacteria. For continued incubation, the killing medium was replaced by maintenance medium containing 10 μg/ml of gentamicin. At indicated time points, cells were lysed either by hypotonic lysis (J774-A.1 cells) or by using a solution of freshly prepared 0.5% sodium deoxycholate in phosphate-buffered saline (MDCK cells), and the number of intracellular bacteria was determined based on the CFU counts of viable bacteria (17).

TABLE 2. Gene-specific primers for PCR controls

Primer	Sequence
Upstream *trxA*	5′-CGAAGTTAACATCAGCACCTCG
Upstream *trxB*	5′-ACGTCGCTGTCGCGGTCGTTCA
Upstream *trxC*	5′-ATCCCGCGCGTAGCGGGACGTC
Upstream *dsbC*	5′-TCCGCGGGCATGAGTGCTGACA
Upstream *gshA*	5′-CAGTCGATGGCGATATCCAGCA
Upstream *grxA*	5′-CCTGTTTAGCCCACCGGCGAAAA

Apoptosis assay. Cells infected with wild-type or the Δ*trxA* mutant bacteria were analyzed for induction of apoptosis 16 h postinfection using an ELISA^{PLUS} cell death detection kit obtained from Roche. This assay was based on determination of enrichment of histone-associated DNA fragments in the cytoplasm. All reagents required were supplied with the kit, and the assay was performed according to the manufacturer's instructions.

Immunoblotting. LB medium cultures of *trxA* mutants complemented with the different redox variants of thioredoxin 1 were grown to the logarithmic phase at 37°C with shaking. A suspension containing 5×10^5 bacteria was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (29), followed by immunoblotting with rabbit anti-thioredoxin 1 or anti-chloramphenicol acetyltransferase serum (catalogue no. T0803 and C-9336, respectively; Sigma). Bound antibody was detected using a peroxidase-conjugated anti-mouse/rabbit secondary antibody (Roche) and a chemoluminescence detection kit (Roche). The blocking solution used was provided with the kit, and all procedures following the transfer to a Hybond P polyvinylidene difluoride membrane (Amersham Pharmacia Biotech) were carried out according to the descriptions provided by the manufacturer of the chemoluminescence detection kit. The Kaleidoscope prestained standard from Bio-Rad was used as a molecular mass marker.

Mouse infection. In vivo competition experiments were carried out using 6- to 8-week-old female BALB/c mice (Taconic, Denmark). The mice were infected intraperitoneally with mutant and wild-type bacteria at a ratio of 1:1 (17). The total load was adjusted to 10^4 bacteria per mouse. Three or four mice from each group were sacrificed at 24 h and 72 h postinfection. The mutant/wild-type ratios in livers and spleens were determined by comparing the CFU counts of viable bacteria. Mutants were distinguished from wild-type bacteria either on the basis of the kanamycin resistance conferred by the replacement cassette or on the basis of the expression of tetracycline resistance coded for by a Tn*10* element that was inserted into the virulence plasmid pSLT and was neutral for virulence (5). In competition experiments with *trxA* mutants complemented with wild-type thioredoxin 1 or a catalytically inactive variant, the two mutants were distinguished on

the basis of the kanamycin resistance of the *trxA*::Km mutant. In addition, samples were plated on LB agar plates supplemented with chloramphenicol or kanamycin and chloramphenicol to ensure that the complementing plasmids were retained throughout the experiment. Mice were housed at Microbiology and Tumor Biology Center, Karolinska Institutet, Stockholm, Sweden, under normal conditions in accordance with both institutional and national guidelines.

Statistical analysis. Data from cell culture experiments were analyzed by a one-way analysis of variance. This analysis indicated differences between the groups with a P value of <0.0001 in all experiments except the phagocytosis assay with J774-A.1 cells and the invasion assay with MDCK cells, which gave P values of <0.001 and <0.05, respectively. The analysis of variance was followed by a two-sided t test to determine the significance of differences between the different groups. Data from the pPIR-A segregation experiment and the mouse experiments were analyzed by a two-sided t test. All P values from the t test analysis are indicated below. Before the t test was performed, all data from the mouse experiments were transformed to $\log_{10}$ values.

RESULTS

Generation of mutants. With the aim of defining the contributions of the thioredoxin and glutathione/glutaredoxin systems to virulence-associated characteristics of *S. enterica* serovar Typhimurium, we separately replaced the genes coding for thioredoxin 1 (*trxA*), thioredoxin 2 (*trxC*), thioredoxin reductase (*trxB*), and the periplasmic thiol-disulfide reductase DsbC (*dsbC*) with a kanamycin resistance cassette. Likewise, we separately replaced the genes coding for the γ-glutamylcysteine synthetase (*gshA*), which is involved in glutathione synthesis, and glutaredoxin 1 (*grxA*). All mutants were generated in the *S. enterica* serovar Typhimurium ATCC 14028 background using recombinase-assisted allelic replacement technology (16). Finally, as the *rho* gene is located transcriptionally downstream of *trxA* and thus is potentially in expressional connection with *trxA*, we also generated a nonpolar mutation by deleting the kanamycin resistance cassette from the original *trxA* mutant. To distinguish between the different *trxA* mutants, the nonpolar mutant is referred to below as Δ*trxA*, and the other mutant is referred to as *trxA*::Km. The nonpolar Δ*trxA* mutant was subsequently used to generate a Δ*trxA trxC*::Km double mutant. Details concerning mutagenesis are described in Materials and Methods.

Mutational inactivation of *trxA*, *trxB*, *trxC*, *grxA*, or *dsbC* does not affect growth in vitro. Apart from being induced upon stress, both the thioredoxins and glutathione/glutaredoxins also have housekeeping functions. One example, emphasizing the necessity to screen mutants depleted of components of these systems for growth defects, is the role of thioredoxins in the reduction of ribonucleotide reductase (1). In order to probe the mutants for potential general growth defects, wild-type and mutant ATCC 14028 strains were grown in rich medium (LB medium) and in a liquid pH 5.8 Casamino Acids-supplemented minimal medium (MM5.8). The latter growth medium was designed to mimic the intravacuolar environment in which *Salmonella* replicates during infection of macrophages (26) and thus was a suitable control for the cell infection experiments described below. In these assays all mutants except the *gshA* mutant, which had a slightly lower growth rate in MM5.8, grew with the same kinetics as the parental wild-type strain (data not shown). Additionally, the wild type and all mutants grew in M9 minimal medium (data not shown), indicating that the mutations did not generate any auxotrophies.

Nevertheless, when the mutants competed against the wild type, a small but significant growth defect was discovered for the *gshA* mutant in LB medium. After 16 h of incubation at 37°C, the mutant/wild-type ratio decreased from 1.0 to 0.6 ($n = 3$; $P < 0.05$). For comparison, when the *trxA*::Km mutant competed against the wild type, the mean ratio remained the same (1.1 at both the zero time point and after 16 h of incubation; $n = 3$; $P > 0.05$).

TABLE 3. MIC of oxidative and nitrosative substances

Strain	MIC (mM)									
	DEM		GSNO			Paraquat		H_2O_2		
	LB medium, stationary/exponential; MM5.8, stationary	MM5.8, exponential	LB medium, stationary/exponential	MM5.8, stationary	MM5.8, exponential	LB medium, stationary/exponential	MM5.8, stationary/exponential	LB medium, stationary	LB medium, exponential	MM5.8, stationary/exponential
ATCC 14028	30	30	>6	3	3	0.39	0.39	1.1	3.3	30
Δ*trxA*	30	30	>6	6	>6	0.39	0.39	1.1	3.3	30
Δ*gshA*	30	3	>6	3	3	0.39	≤0.039	1.1	3.3	0.36

Sensitivity to radical stress in vitro. We began by evaluating the role of the thioredoxin and glutathione/glutaredoxin systems in *S. enterica* serovar Typhimurium by screening mutant and wild-type bacteria for differences in the MIC of hydrogen peroxide (H_2O_2), DEM, paraquat, or the nitric oxide donor compound GSNO. Bacteria were grown in LB medium or MM5.8 to either the late logarithmic or stationary phase before they were exposed to the different compounds. Surprisingly, in these analyses all of the mutants except the *gshA* mutant exhibited overall maintained resistance to the substances in the concentration range tested, and the *trxA*::Km mutant even exhibited moderately increased resistance to GSNO when it was grown in MM5.8 (Table 3). However, the MIC of DEM, paraquat, and H_2O_2 for the *gshA* mutant were decreased when the bacteria were grown in MM5.8.

Mutants that lack thioredoxin 1 are phenotypically invasive but defective for growth in epithelial MDCK cells. When *S. enterica* serovar Typhimurium is grown in LB medium to the early stationary phase, this bacterium becomes highly invasive due to expression of the *Salmonella* pathogenicity island 1 (SPI1) type III secretion system (TTSS) and concomitant effector proteins that mediate bacterial invasion of a variety of host cells (14). As the periplasmic thiol-disulfide oxidase DsbA has been implicated in virulence and in the function of TTSSs (36), we next studied the abilities of the mutants to invade and replicate in epithelial MDCK cells. Bacteria that were grown to the early stationary phase were seeded on MDCK cells, and samples were taken 2 and 16 h postinfection for enumeration of viable intracellular bacteria.

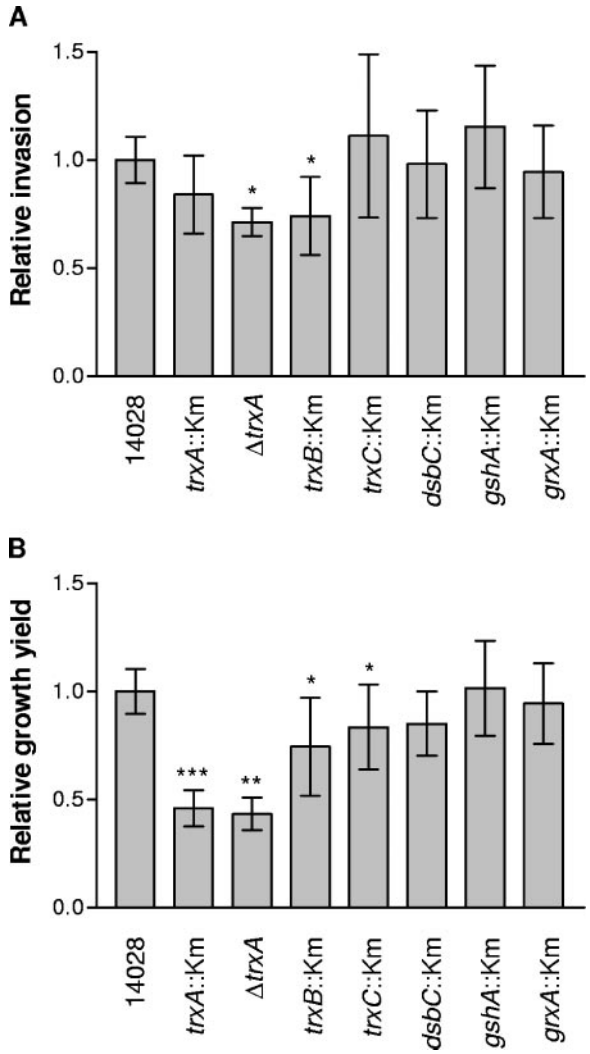

FIG. 2. Invasion by (A) and growth of (B) the wild type and the different mutants of *S. enterica* serovar Typhimurium in MDCK cells. MDCK cells were infected with phenotypically invasive bacteria. At 1 h postinfection, cells were treated with gentamicin (50 μg/ml) for 45 min to kill extracellular bacteria. At 2 h and 16 h postinfection cells were lysed in order to measure invasion efficacy (CFU at 2 h/CFU seeded on the cells) and growth yields (CFU at 16 h/CFU at 2 h), respectively. All values were normalized to the wild-type value. The error bars indicate standard deviations ($n \geq 3$). One asterisk indicates that the P value is <0.05, two asterisks indicate that the P value is <0.001, and three asterisks indicate that the P value is <0.0001, as determined by the Student t test.

In these experiments, the mutants exhibited only minor alterations in the invasion efficacy compared to the invasion efficacy of the wild type (Fig. 2A). While the small reductions in invasion efficacy exhibited by the Δ*trxA* and *trxB*::Km mutants were statistically significant, considering the magnitude of the decreases, they are unlikely to be biologically relevant. In correlation with these data, we did not detect any significant differences between mutant and wild-type bacteria in expression or secretion of SPI1 effector proteins, as determined by a sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of culture supernatants from phenotypically invasive bacteria (data not shown). When we compared the growth yields, the *trxB* and *dsbC* mutants exhibited minor but statistically significant reductions in growth. The *trxA*::Km mutant, however, exhibited a more pronounced decrease in growth than the wild type exhibited (Fig. 2B). As indicated above, the genome sequence of *S. enterica* serovar Typhimurium LT2 includes a reading frame just downstream of *trxA*. In order to exclude any polar effects of the kanamycin resistance replacement cassette, this cassette was excised to create a nonpolar *trxA* deletion. In invasion and intracellular replication experiments the nonpolar Δ*trxA* mutant behaved like the original *trxA*::Km mutant, exhibiting an almost identical, statistically significant reduction in the growth yield (Fig. 2A and B).

Mutants that lack thioredoxin 1 or thioredoxin reductase have a growth defect in macrophage-like J774-A.1 cells. We next assessed whether any of the mutants were affected either in the efficacy of phagocytosis or in subsequent intracellular bacterial proliferation in the murine macrophage-like cell line J774-A.1. When J774-A.1 cells were infected with phenotypically noninvasive bacteria opsonized with preimmune mouse serum, all mutants exhibited a small decrease in phagocytosis (or early survival) compared to the wild-type strain (Fig. 3A). Strikingly, with the prominent exception of the *trxA* and *trxB* mutants, none of the mutants exhibited any significant replication defects (Fig. 3B). In fact, for the *trxC*, *dsbC*, and *grxA* mutants there were more-than-twofold increases in the growth yields compared to the wild type, which were statistically significant (Fig. 3B).

We also assessed the growth of a Δ*trxA trxC*::Km double mutant to evaluate whether the increase in the growth yield associated with the *trxC* mutation could compensate for the growth defect of a *trxA* mutant. However, the growth yield of the Δ*trxA trxC*::Km double mutant was similar to that of the *trxA* single mutants (Fig. 3A and B).

Wild-type and *trxA* mutant bacteria do not differ in apoptotic potential. Phenotypically invasive *S. enterica* serovar Typhimurium is known to cause a relatively rapid SPI1-dependent apoptotic response in infected monocytic cells (37). Although phenotypically noninvasive bacteria were used for infection of J774.A-1 cells, we wanted to ensure that the decreased growth yields obtained with the *trxA* mutants were not a consequence of increased bacterium-mediated cytotoxicity. To settle this issue, both J774.A-1 and MDCK cells were infected with wild-type and thioredoxin 1-deficient *S. enterica* serovar Typhimurium, and enrichment of DNA-histone complexes in the cytosol of infected cells was used as a readout of apoptosis 16 h postinfection. In this analysis, some apoptosis was induced in infected cells compared to noninfected controls. However, there was no significant difference between cells infected with wild-type bacteria and cells infected with *trxA*::Km mutant bacteria (Fig. 4A and B).

A *trxA* mutant has a lower replication rate in J774-A.1 macrophage-like cells. We next compared the growth rates of wild-type *S. enterica* serovar Typhimurium and the *trxA*::Km mutant inside J774-A.1 cells. Replication was assayed by monitoring the segregation of the pPIR-A plasmid (13, 44). In addition to an ampicillin resistance marker, this plasmid harbors a temperature-sensitive replicon that makes it unable to replicate at 37°C. This causes the plasmid to segregate as a function of bacterial cell division at 37°C, and consequently determination of the proportion of ampicillin resistance among bacterial pro-

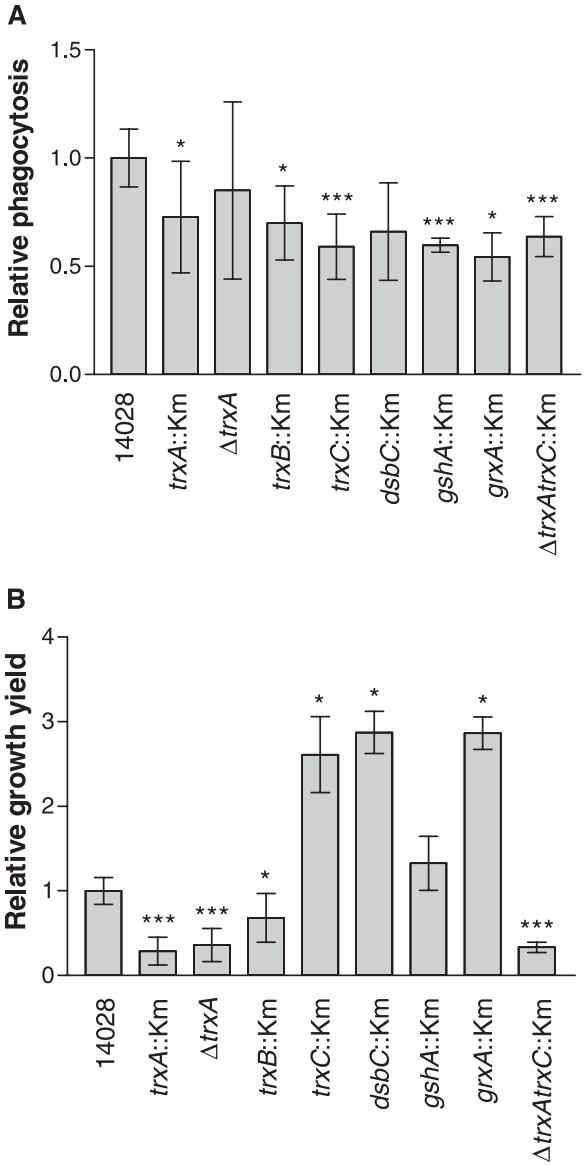

FIG. 3. Efficacy of phagocytosis (A) and intracellular proliferation (B) of wild-type and mutant *S. enterica* serovar Typhimurium bacteria in J774-A.1 macrophage-like cells. Cells were infected with phenotypically noninvasive bacteria opsonized with 10% preimmune mouse sera. At 1 h postinfection, cells were treated with gentamicin (50 μg/ml) for 45 min to kill the extracellular bacteria. At 2 h and 16 h postinfection cells were lysed in order to measure phagocytosis efficacy (CFU at 2 h/CFU seeded on the cells) and growth yields (CFU at 16 h/CFU at 2 h), respectively. All values were normalized to the wild-type value. The error bars indicate standard deviations ($n \geq 3$). One asterisk indicates that the P value is <0.05, and three asterisks indicate that the P value is <0.0001, as determined by the Student t test.

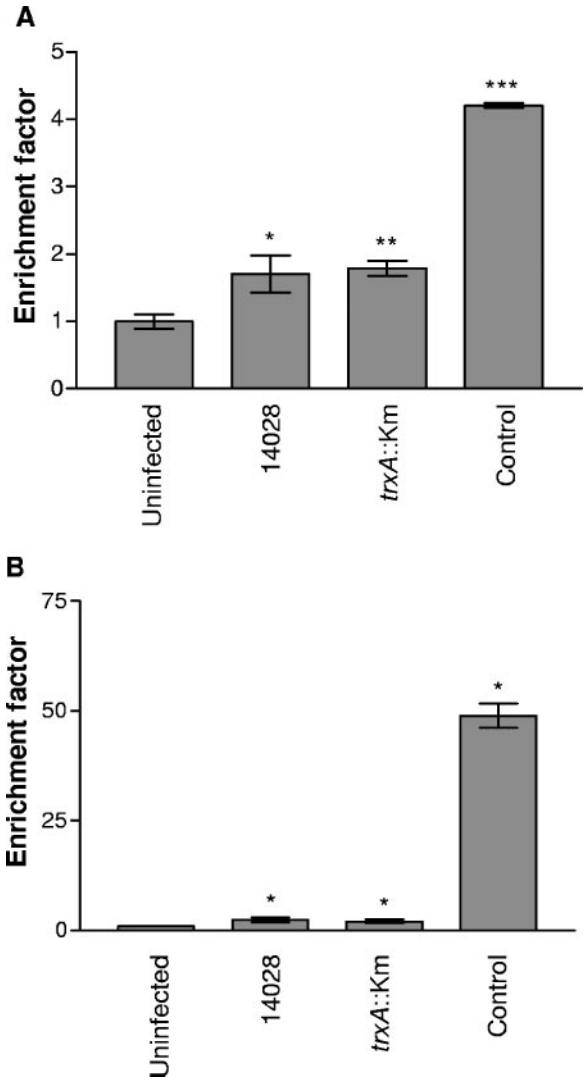

FIG. 4. Induction of apoptosis by wild-type and *trxA* mutant bacteria in J774-A.1 and MDCK cells. Enrichment of DNA-histone complexes in the cytosol of infected cells, as measured by an ELISA, was used as an indication of apoptosis. The enrichment factor was calculated as follows: absorbance of the sample/absorbance of the uninfected cells. Purified DNA-histone complexes provided with the ELISA kit were used as the positive control. A significantly longer incubation time with the ELISA substrate was required in panel B than in panel A before color development was detected. This resulted in a more intense color for the positive control in panel B than in panel A and is the explanation for the apparently large difference in enrichment factor between the positive control in panel A and panel B. The error bars indicate standard deviations ($n = 3$). One asterisk indicates that the P value is <0.05, two asterisks indicate that the P value is <0.001, and three asterisks indicate that the P value is <0.0001, as determined by the Student t test.

genitors can be used to measure bacterial replication. Hence, if the lower bacterial yield obtained for the *trxA*::Km mutant was due to a reduced growth rate, the proportion of ampicillin-resistant bacteria should have decreased during host cell infection but to a lesser extent than for the wild type, whereas if the lower bacterial yield was caused by increased killing, the proportion of ampicillin resistance should have decreased to the same extent for mutant and wild-type bacteria.

Thus, J774-A.1 cells were infected with wild-type or *trxA*::Km mutant bacteria carrying the pPIR-A plasmid. At 16 h postinfection bacteria were recovered from the cells and used to infect a new set of cells without passage of the bacteria on ordinary growth medium, and bacteria were recovered after another 16 h of incubation to obtain the apparent 32-h. The bacterial growth yields were determined by comparing the

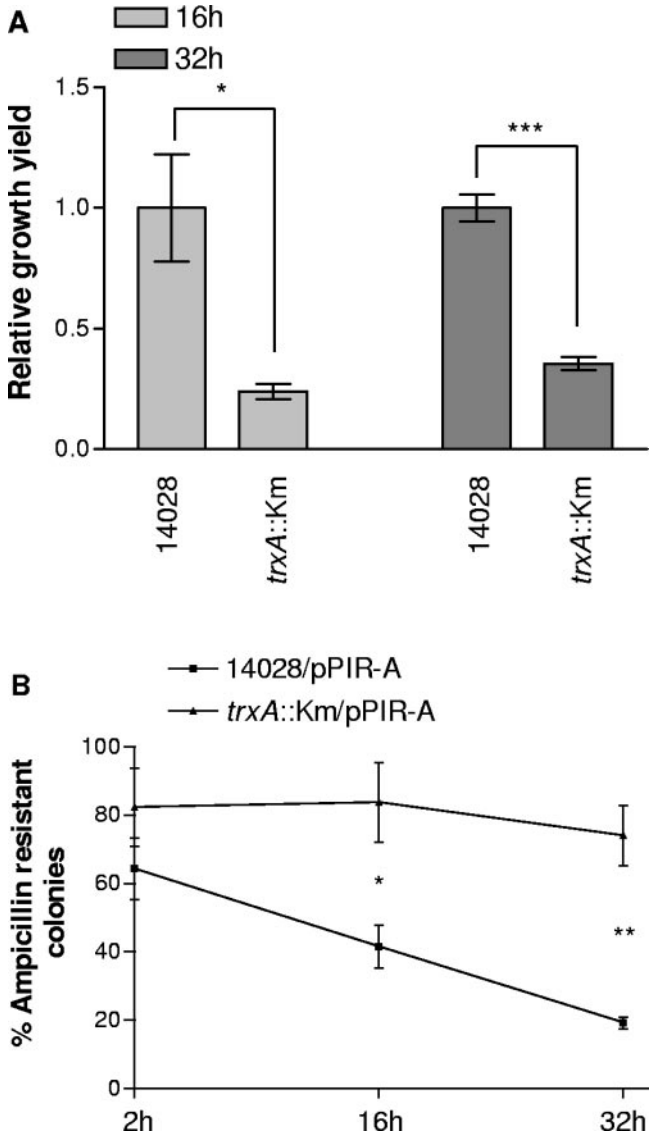

FIG. 5. Intracellular replication efficacy of *S. enterica* serovar Typhimurium wild type and *trxA* mutant in macrophage-like J774-A.1 cells, as measured by segregation of the temperature-sensitive plasmid pPIR-A. Growth yields (A) and percentage of viable ampicillin-resistant bacteria (B) were determined. In panel A, the value for the *trxA* mutant was normalized to the wild-type value at each time point. The error bars indicate standard deviations ($n \geq 3$). One asterisk indicates that the P value is <0.05, two asterisks indicate that the P value is <0.001, and three asterisks indicate that the P value is <0.0001, as determined by the Student t test.

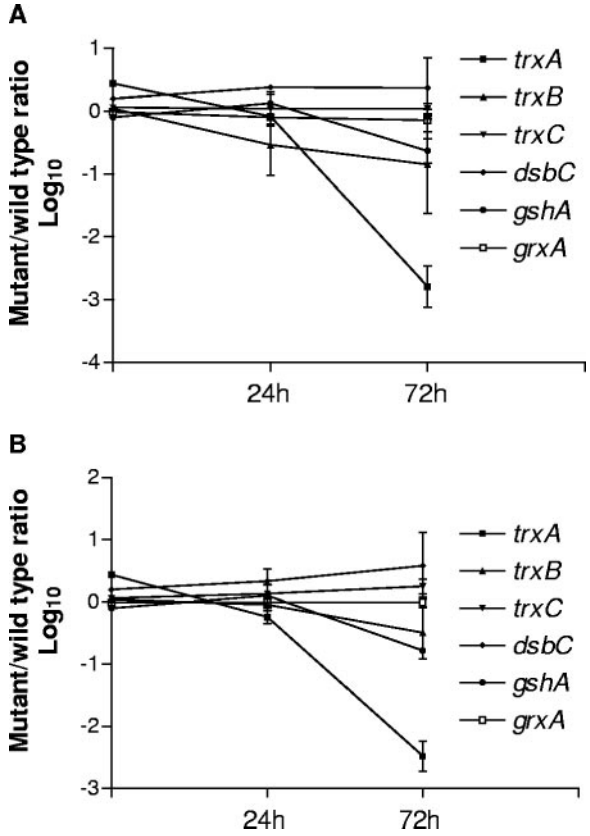

FIG. 6. In vivo fitness and virulence of the various mutants compared to wild-type *S. enterica* serovar Typhimurium. BALB/c mice were infected intraperitoneally with mixtures of wild-type and mutant bacteria at a 1:1 ratio (the total dose was 10^4 bacteria per mouse). Mice were sacrificed on day 1 (24 h) and day 3 (72 h) postinfection, and the proportions of wild-type and mutant bacteria in the livers (A) and spleens (B) were determined by CFU counts in homogenized tissues. The error bars indicate standard deviations ($n = 3$). Statistical significance was observed only for the small decrease in the mutant/wild-type ratio for the *gshA* mutant and for the more pronounced decrease for the *trxA*::Km mutant ($P < 0.05$, as determined by the Student t test).

CFU counts at 2 and 16 h. In parallel, the percentages of viable ampicillin-resistant bacteria were determined for the various samples. In this assay, the growth index for the wild-type strain was significantly higher that that for the *trxA*::Km mutant (Fig. 5A), as anticipated from the growth experiment with macrophage-like J774-A.1 cells described above (Fig. 3B). When the proportion of ampicillin resistance was measured, the pPIR-A plasmid segregated more efficiently in wild-type bacteria than in the *trxA*::Km mutant (Fig. 5B). This implied that the lower intracellular growth yield of the *trxA*::Km mutant was due at least in part to a slower intracellular generation time.

Thioredoxin 1 is required for *S. enterica* serovar Typhimurium virulence in BALB/c mice. The ability of *S. enterica* serovar Typhimurium to replicate inside host cells is strongly associated with the ability to cause disease (20). Therefore, we performed experiments to determine the in vivo competition between wild-type bacteria and the mutants deficient in individual components of the thioredoxin and glutathione/glutaredoxin pathways. BALB/c mice were infected intraperitoneally with mixtures of wild-type and mutant bacteria at a 1:1 ratio, using a total dose of 10^4 bacteria per mouse. Groups of mice were sacrificed on day 1 (24 h) and on day 3 (72 h) postinfection, and the proportions of wild-type and mutant bacteria in the liver and spleen were determined by CFU counts in homogenized tissues. To distinguish between wild-type and mutant bacteria, samples were plated both on ordinary LB agar plates and on plates supplemented with kanamycin. On day 1 postinfection, the mutant/wild-type ratios were close to 1, imply-

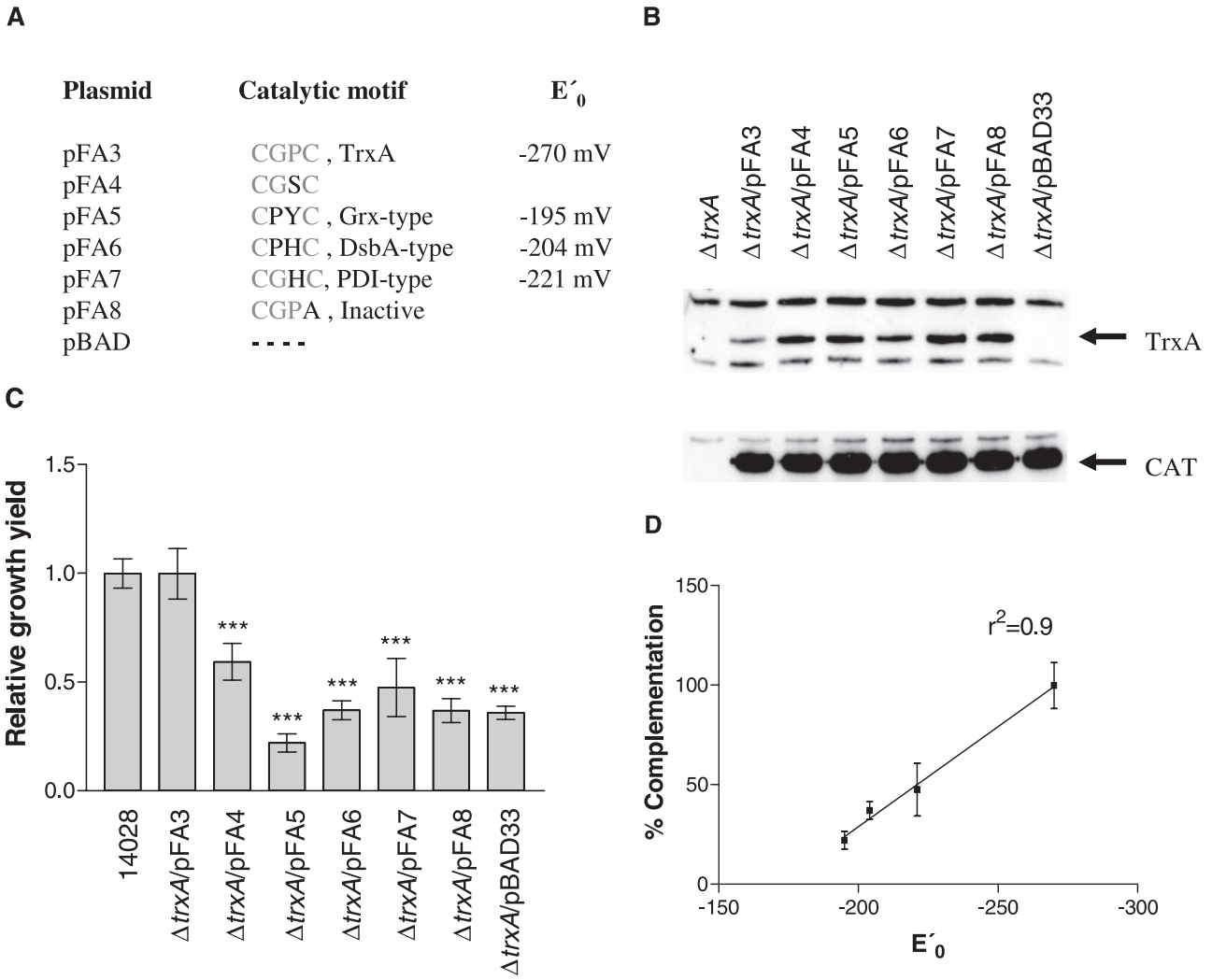

FIG. 7. Correlation between the reductivity of thioredoxin 1 and the ability to complement a *trxA* mutant. (A) Catalytic motifs and reduction potentials of the different constructs. (B) Immunoblotting of *trxA* mutants expressing either of the catalytic variants in *trans* with anti-TrxA and anti-CAT as an internal control showed that there was equal expression of the different variants. (C) J774-A.1 cells were infected with thioredoxin 1-deficient bacteria complemented with the different redox variants, and the abilities of these constructs to complement the growth defect were analyzed at 16 h postinfection. The data obtained for pFA3, pFA5, pFA6, and pFA7 were used in a regression analysis (D), which showed that there was a straight correlation between redox potential and the ability to complement ($P < 0.0001$). All values in panel C were normalized to the wild-type value. The error bars indicate standard deviations ($n \geq 3$). Three asterisks indicate that the P value is <0.0001, as determined by the Student t test.

ing that all six mutants translocated to the liver (Fig. 6A) and spleen (Fig. 6B) to the same extent as the wild type. However, at the later time point (72 h), there was a small decrease in the virulence of the *gshA* mutant. The *trxA*::Km mutant exhibited a drastic decrease in the mutant/wild-type ratio, implying that there was strong attenuation of virulence (Fig. 6A and B). This experiment was repeated twice with the *trxA*::Km mutant, and similar results were obtained, which verified the importance of TrxA for in vivo virulence. The data for the *trxA*::Km mutant shown in Fig. 6 are data from one representative experiment.

We also assessed the virulence of the nonpolar Δ*trxA* mutant by determining the mutant/wild-type ratio in BALB/c mice. To distinguish between mutant and wild-type bacteria, the wild type was tagged with a neutral Tn*10* insertion (5). There was also a pronounced decrease in the mutant/wild-type ratio when the nonpolar mutant was used, with less than 1% of the mutant recovered in relation to the Tn*10*-tagged wild type at 72 h postinfection (data not shown). Finally, as inactivation of *trxC* resulted in increased bacterial replication in murine macrophage-like J774-A.1 cells, we also determined the ratio of the Δ*trxA trxC*::Km double mutant to the wild-type strain in BALB/c mice. In these experiments, however, the mutant/wild-type ratio was below the detection limit at 72 h postinfection (data not shown). Thus, thioredoxin 1 does contribute to the in vivo virulence of *S. enterica* serovar Typhimurium in the mouse infection model.

The contribution to intracellular growth and in vivo virulence by thioredoxin 1 depends on its catalytic redox activity. Thioredoxin 1 is an evolutionarily conserved reductase, in which the catalytic active site is characterized by a Cys-X-X-

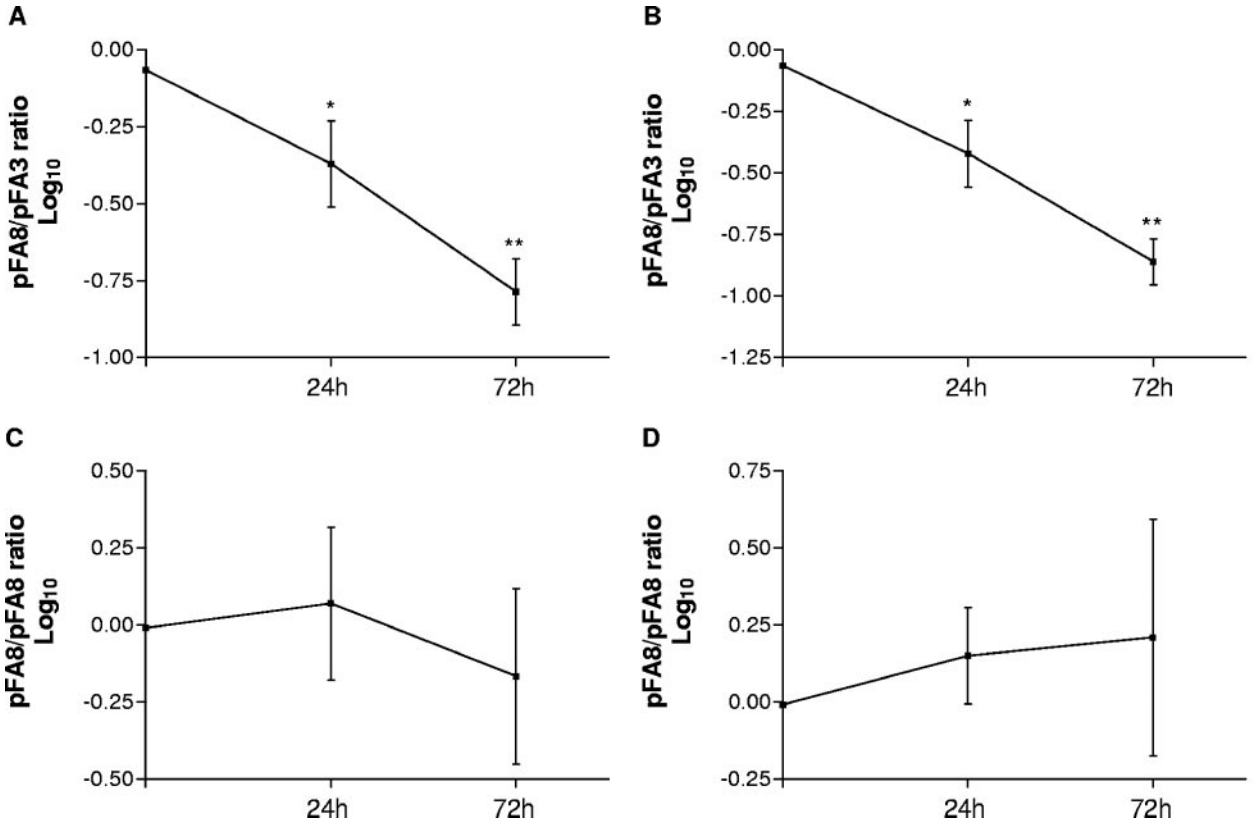

FIG. 8. In vivo virulence depends on the redox activity of thioredoxin 1. BALB/c mice were infected intraperitoneally with mixtures of either the Δ*trxA* mutant complemented with pFA3 (wild-type *trxA*) and the *trxA*::Km mutant complemented with pFA8 (catalytically inactive *trxA*) or both the Δ*trxA* and *trxA*::Km mutants complemented with pFA8 at a 1:1 ratio (the total dose was 10^4 bacteria per mouse). Mice were sacrificed on day 1 (24 h) and day 3 (72 h) postinfection, and the proportions of pFA8- and pFA3-containing bacteria in the livers (A and C) and spleens (B and D) were determined by CFU counts in homogenized tissues. The error bars indicate standard deviations ($n = 4$). One asterisk indicates that the P value is <0.05, and two asterisks indicate that the P value is <0.001, as determined by the Student t test.

Cys motif. In addition, recent observations defined a protein chaperone function for thioredoxin 1 that is not related to its catalytic activity (25). As shown by the data in Table 3 and Fig. 2 and 3, the sustained in vitro tolerance to oxidative substances of the *trxA*::Km mutant did not correlate with the observed intracellular replication defect. We therefore wanted to establish to what degree the intracellular replication depended on the redox activity of thioredoxin 1. To study this, we used a collection of *trxA* constructs expressing either wild-type *E. coli* K-12 thioredoxin 1 or thioredoxin 1 catalytic site mutants with decreased reducing capabilities or with a complete lack of redox activity due to single amino acid substitutions (Fig. 7A). The choice of this series of constructs was justified by the fact that the primary structures of the *E. coli* K-12 and *S. enterica* serovar Typhimurium LT2 thioredoxin 1 proteins are identical (7, 35).

To ensure that thioredoxin 1 was expressed to the same extent by the different constructs and to exclude any sensitization to proteolytic cleavage possibly conferred by the amino acid substitutions, the levels of thioredoxin 1 in Δ*trxA* mutant bacteria complemented with the different variants were analyzed. The vectors that expressed the thioredoxin 1 variants also encoded chloramphenicol acetyltransferase (CAT), which functioned as an internal control in this analysis. Immunoblotting experiments for thioredoxin 1 and CAT verified that the levels of thioredoxin 1 expressed by the various constructs were comparable (Fig. 7B).

Having eliminated the possibility that there were any differences in expression of the different variants of thioredoxin 1, we next studied the effects of complementation of Δ*trxA* mutant bacteria with the thioredoxin 1 variants during infection of J774-A.1 cells. In this assay complementation with pFA3, which codes for wild-type thioredoxin 1, completely restored the intracellular replication of the Δ*trxA* mutant, while pFA8, which codes for the noncatalytic variant, did not exhibit any complementation ability (Fig. 7C). Interestingly, for the remaining catalytically active variants of thioredoxin 1, there appeared to be a correlation between redox potential and the degree of complementation. In fact, the growth yield for the strain complemented with the least reducing variant of thioredoxin 1, coded for by pFA5, was lower than the growth yield obtained with the empty cloning vector pBAD33 (Fig. 7C). A regression analysis showed that there was a straight correlation between redox potential and complementation ability (Fig. 7D).

Next, we wanted to establish whether the decreased in vivo virulence of the *trxA* mutants also depended on the redox activity of thioredoxin 1. Thus, BALB/c mice were infected

intraperitoneally with a mixture of the Δ*trxA* mutant complemented with pFA3 (wild-type *trxA*) and the *trxA*::Km mutant complemented with pFA8 (catalytically inactive *trxA*) or a mixture of both Δ*trxA* and *trxA*::Km complemented with pFA8 at a 1:1 ratio; the total dose was 10^4 bacteria per mouse. To distinguish between the two *trxA* mutants and to ensure that the complementing plasmids were retained at a high efficiency, samples were plated on ordinary LB agar plates and on plates supplemented with kanamycin, chloramphenicol, or kanamycin and chloramphenicol. In these experiments the pFA8/pFA3 ratio decreased continuously from day 0 to day 3 in both the liver and the spleen (Fig. 8A and B), whereas the pFA8/pFA8 ratio remained fairly constant (Fig. 8C and D). This indicated that in vivo bacterial virulence at least partially depended on the redox activity of thioredoxin 1.

DISCUSSION

The ability to cope with oxidative damage, the ability to maintain proper formation of protein disulfide bonds, and the required reduction state of protein sulfhydryl groups must be major challenges for any cell adapted to an aerobic environment. The biochemical functions of the thioredoxin and glutathione/glutaredoxin systems (Fig. 1) imply that these systems have roles in cellular redox shuffling and protection against oxidative stress and, through the connection with periplasmic disulfide isomerases, roles in the formation and maintenance of proper disulfide bonding of proteins outside the reducing cytosol environment (3, 23, 51). Deficiencies in the thioredoxin and glutathione/glutaredoxin pathways have been reported to sensitize bacteria such as *E. coli*, *Helicobacter pylori*, and *Rhodobacter sphareoides* to hydrogen peroxide in vitro (15, 31, 51), whereas inactivation of a homologue of glutathione peroxidase (*gpxA*) in *Neisseria* has been reported to cause increased sensitivity to paraquat (38). Additionally, in *E. coli* glutaredoxin has been suggested to act as an "S-nitrosyl sink" in protection against nitric oxide stress (22).

The species *S. enterica* includes a number of serovariants that are facultative intracellular parasites and that characteristically target phagocytic cells. Two major defense mechanisms expressed by professional phagocytes are the production of reactive oxygen and nitrogen species (33, 54, 55). Hence, it is not surprising that virulent *S. enterica* serovar Typhimurium has been found to express genes that mediate an antioxidative defense. SoxS is known to mediate tolerance to paraquat in vitro (19), whereas expression of the superoxide dismutase SodCI is induced upon infection of macrophage-like cells and is required for virulence in mice (18, 53). When replicating in phagocytic cells, *S. enterica* serovar Typhimurium is reported to exclude the phagocyte NADPH oxidase and the inducible nitric oxide synthase from the *Salmonella*-containing vacuole in an SPI2-dependent manner (12, 56). Moreover, the SPI2 TTSS has been reported to depend on the periplasmic oxidoreductase DsbA for proper function (36). Thus, one would expect active participation of the thioredoxin and glutathione/glutaredoxin systems in the protection of salmonellae against oxidative damage, in inappropriate formation of disulfide bonds, and, due to their function as electron donors, in the pathway for disulfide bond isomerization in the periplasm, as well as in the expression of complex surface-associated virulence factors, such as the type III secretion systems (36).

When sensitization of *trxA*, *trxB*, *trxC*, *gshA*, *grxA*, and *dsbC* mutants of *S. enterica* serovar Typhimurium to oxidative and nitric oxide donor substances in vitro was studied, increased sensitivity to oxidative substances was observed only for the *gshA* mutant deficient in γ-glutamylcysteine synthetase (Table 3). During infection of epithelial MDCK cells, small but statistically significant decreases in internalization were observed for the Δ*trxA* and *trxB* mutants (Fig. 2A). In parallel, small but statistically significant decreases in intracellular replication were observed for the *trxB* and *trxC* mutants. However, the decrease in replication efficacy was small compared to that of the wild-type strain. In contrast, there was a more pronounced and significant decrease in the replication efficacy of the *trxA* mutants in MDCK cells (Fig. 2B).

When mutants were probed for bacterial uptake and replication in macrophage-like J774-A.1 cells, small but statistically significant decreases in initial uptake or early survival were observed for the *trxA*, *trxB*, *trxC*, *gshA*, and *grxA* mutants (Fig. 3A). The *trxA* mutants showed a statistically significant decrease in intracellular growth, whereas the *trxB* mutant displayed a small decrease in intracellular replication, comparable to its behavior in MDCK cells (Fig. 3B). Surprisingly, significant increases in replication of the *trxC*, *dsbC*, and *grxA* mutants inside murine macrophage-like J774-A.1 cells were observed (Fig. 3B). Subsequent experiments in which the replication efficacies of wild-type and *trxA*::Km bacteria in murine macrophage-like J774-A.1 cells were compared showed that the decreased yield of *trxA*::Km mutant bacteria resulted at least in part from a significantly lower intracellular replication rate. Additionally, when the fitness of the various mutants in BALB/c mice was studied, we observed a small decrease in the virulence of the *gshA* mutant, whereas there were pronounced degrees of attenuation only for the *trxA* mutants. In this respect, the behavior of the *trxA* mutants resembled that of *purE* mutants of *S. enterica* serovar Typhimurium. The replication of *pur* mutants is affected in epithelial MDCK cells (30) and murine macrophage-like J774-A.1 cells (10), as well as in BALB/c mice (43).

Thioredoxin 1 is an evolutionarily conserved protein, and the primary structures of the *E. coli* and *S. enterica* serovar Typhimurium LT2 proteins are identical and have a characteristic Cys-X-X-Cys motif that defines the catalytic site and the redox activity. Thioredoxin 1 was initially identified on the basis of its ability to provide redox equivalents for ribonucleotide reductases, which are enzymes that generate deoxyribonucleotides essential for bacterial DNA replication (1). In *E. coli*, thioredoxin 1 also maintains resistance to oxidative stress, which implies that the relatively strong reducing potential of this protein (E'_0, −270 mV) also restores appropriate cytoplasmic disulfide status (3). However, at least two lines of evidence indicate that thioredoxin 1 has a function that does not rely on reduction of cysteines in target proteins. First, as reported by Kern et al. (25), *E. coli* thioredoxin 1 was found to have a protein chaperone function in vitro for denatured citrate synthetase, and this capacity was completely active even when an oxidized form of thioredoxin 1 was used (25). Second, by using a protein capture approach, 80 proteins were identified in *E. coli* that associated with thioredoxin 1, 4 of which are

enzymes involved in detoxification and oxidative stress responses. Yet 20 of the proteins, including a manganese-dependent superoxide dismutase, did not have cysteines in their primary structure, which made them incapable of interacting with thioredoxin 1 through the formation of mixed disulfides (28). Thus, we found that it was necessary to establish whether the virulence-associated characteristics of thioredoxin 1 were connected to its catalytic activity. By using different redox variants of thioredoxin 1 in complementation assays, we showed that there is a clear correlation between redox activity and restoration of intracellular growth of the Δ*trxA* mutant in macrophage-like J774-A.1 cells. Wild-type thioredoxin 1 with an E'_0 of −270 mV completely restored the intracellular replication efficacy of the Δ*trxA* mutant, while a catalytically inactive variant of thioredoxin 1 did not confer any complementation. In contrast, the variant with an E'_0 of −195 mV, which was more oxidative, actually decreased the intracellular growth potential of the Δ*trxA* mutant. This implies that the replication efficacy of *S. enterica* serovar Typhimurium in macrophage-like J774-A.1 cells depends on the reductivity of thioredoxin 1. Likewise, complementation for virulence in BALB/c mice occurred when redox-active wild-type thioredoxin 1 was used but not when the catalytically inactive variant was used. These observations clearly showed that the redox activity of thioredoxin 1 does contribute to virulence.

In this context, the observed increased intracellular replication of the *trxC*, *grxA*, and *dsbC* mutants in macrophage-like J774-A.1 cells deserves some attention. It has been reported that inactivation of *trxA* in *E. coli* increases the expression of glutaredoxins, while inactivation of thioredoxins or glutaredoxins increases catalase activity (45, 57). Thus, compensatory cross-regulation between the thioredoxin and glutathione/glutaredoxin pathways could explain maintenance against oxidation- or nitrosation-mediated killing of the *trxC*, *grxA*, and *dsbC* mutants. However, the results of immunoblotting experiments did not suggest that there is any compensatory upregulation in the expression of thioredoxin 1 in the *trxC*, *grxA*, and *dsbC* mutants (data not shown). Therefore, considering the impact of redox-active thioredoxin 1 for intracellular replication, one could envision an alternative scenario in which thioredoxin 1, when there is a shortage of NADPH, competes with thioredoxin 2 and DsbC for reducing equivalents and that depletion of either thioredoxin 2 or DsbC leaves thioredoxin 1 with more catalytic capacity to promote intracellular replication. This suggestion is also consistent with the slightly decreased intracellular replication efficacy of the *trxB* mutant, since thioredoxin reductase shuffles redox equivalents from NADPH to thioredoxin 1 and thioredoxin 2 (Fig. 1), and with the observation that the increased intracellular replication of the *trxC* mutant does not occur with a Δ*trxA trxC*::Km double mutant. Furthermore, as both GshA and thioredoxin reductase use NADPH, a lack of the redox-consuming thioredoxin 1 could explain the observed small increase in GSNO tolerance of the *trxA*::Km mutant (Table 3).

To summarize, we showed that in *S. enterica* serovar Typhimurium thioredoxin 1 has a substantial effect on bacterial intracellular replication and virulence, yet this role of thioredoxin 1 does not correlate with tolerance to oxidative stress in vitro. In contrast, in vitro tolerance to oxidative stress strongly depends on the synthesis of glutathione.

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